Method of identifying fungicidally active compounds based on fungal mevalonate kinases

The invention relates to a method of identifying fungicides, to the use of mevalonate kinase for identifying fungicides, and to the use of mevalonate kinase inhibitors as fungicides.

Undesired fungal growth which, every year, results in considerable damage in agriculture can be controlled by the use of fungicides. The demands which fungicides have to meet with regard to their efficacy, costs and above all their environmental compatibility have become more and more stringent. There is therefore a need for novel substances, or classes of substances, which can be developed into potent and environmentally compatible novel fungicides. In general, the usual procedure is to search for such novel lead structures in greenhouse tests. However, such tests require a great deal of labor and are expensive. Accordingly, the number of substances which can be tested in the greenhouse is limited. An alternative to such tests is the use of what are known as high-throughput screening methods (HTS methods). Here, a large number of individual substances are assayed in an automated procedure with regard to their effect on cells, individual gene products or genes. If an effect is detected for certain substances, they can be studied in conventional screening methods and, if appropriate, developed further.

Advantageous novel targets for fungicides which can then be used in the abovementioned HTS methods are frequently searched for in essential biosynthetic pathways. Ideal fungicides are furthermore those substances which inhibit gene products which play a decisive role in the manifestation of the pathogenicity of a fungus.

It was therefore an object of the present invention to identify, and make available, a suitable novel target for potential fungicidally active ingredients and, based thereon, to provide a method which makes possible the identification of modulators of this target, preferably in a high-throughput screening method, thus making possible the identification of novel fungicides.

Figures and sequence listing

Figure 1: Schematic representation of the reaction catalyzed by mevalonate kinase. Mevalonate kinase catalyzes the reaction of (R)-mevalonate and adenosine triphosphate to (R)-5-phosphomevalonate and adenosine diphosphate.

Figure 2: Homology between mevalonate kinases from a variety of fungi. (1) *Saccharomycs cerevisiae*, (2) *Schizosaccharomyces pombe*, (3) *Ustilago maydis*, (4) *Neurospora crassa*, (5) *Magnaporthe grisea*. Frames indicate regions with exactly matching sequences (consensus sequence).

Figure 3: SDS gel showing the course of the heterologous expression of mevalonate kinase in *E. coli* BL21 (DE3). The overexpressed GST fusion protein has a size of 74.5 kDa. Size markers were applied in lanes M. Lane 1: pellet fraction; lane 2: cytoplasmic fraction of the overexpressed mevalonate kinase (3 hours after induction with 100 mM IPTG at 30°C); lane 3: wash fraction after application of the cytoplasmic fraction to the glutathione-Sepharose column; lane 4: elution fraction with purified mevalonate kinase.

Figure 4: Kinetics of the conversion of mevalonate and adenosine triphosphate in the assay with different mevalonate kinase concentrations. 300 µM adenosine triphosphate, 500 µM mevalonate, 300 µM NADH, 400 µM phosphoenol pyruvate, 0.2 U pyruvate kinase and 0.4 U lactate dehydrogenase and different amounts of mevalonate kinase were employed in an assay volume of 50 µl. The mevalonate kinase protein concentrations used can be seen from the figure. The conversion of mevalonate was monitored with reference to the coupled reaction with pyruvate kinase and lactate dehydrogenase. The conversion of ATP by means of mevalonate is monitored by the coupled decrease in absorption at 340 nm (decrease of NADH in coupled reaction).

SEQ ID NO:1 Nucleic acid sequence coding for the mevalonate kinase from *Ustilago maydis*. The sequence shown corresponds to the genomic DNA.

SEQ ID NO: 2 Amino acid sequence of the mevalonate kinase from *Ustilago maydis*.

Definitions

The term "homology" or "identity" is understood as meaning the number of matching amino acids (identity) with other proteins, expressed in percent. The identity is preferably determined by comparing a given sequence with other proteins with the aid of computer programmes. If sequences which are compared with one another differ with regard to their lengths, the identity is to be determined in such a way that the number of amino acids which the shorter sequence shares with the longer sequence determines the percentage of identity. The identity can be determined routinely by means of known and publically available computer programmes such as, for example, ClustalW (Thompson et al., *Nucleic Acids Research 22* (1994), 4673-4680). ClustalW is publically available for example from Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can also be downloaded from different internet pages, inter alia at the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and at the EBI (ftp://ftp.ebi.ac.uk/pub/software/) and at all mirrored internet pages of the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK). If the computer programme ClustalW, Version 1.8, is used for determining the identity between, for example, a given reference protein and other proteins, the following parameters are to be employed: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP. One possibility of finding similar sequences is to carry out sequence database searches. Here, one or more sequences are set as what is known as a query. This query sequence is then compared with sequences which are present in the selected databases, using statistic computer programmes. Such database queries (blast searches) are known to the skilled worker and can be carried out at different providers. If such a database search is carried out for example at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), the standard settings for the specific comparative search should be used. For protein sequence comparisons ("blastp"), these settings are as follows: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1. As the result of such a query, the degree of identity between the query sequence and the similar sequences found in the databases are shown, in addition to other parameters. A protein according to the invention is therefore, in the context of the present invention, to be understood as meaning those proteins which have an identity of at least 50%, preferably of at least 60%, especially preferably of at least 70%, more preferably of at least 80% and in particular of at least 90% when using at least one of the above-described methods for determining the identity.

The term "complete mevalonate kinase" as used in the present context describes a mevalonate kinase which is encoded by a complete coding region of a transcriptional unit comprising the ATG start codon and all information-bearing exon regions of the gene which is present in the organism of origin and which codes for mevalonate kinase, and comprising the signals required for correctly terminating the transcription process.

The term "enzymatic activity of a mevalonate kinase" as used in the present context refers to the ability of a polypeptide to catalyze the above-described reaction, i.e. the conversion of mevalonate and adenosine triphosphate into phosphomevalonate and adenosine diphosphate. The reaction described represents the reaction which is predominantly catalyzed by the polypeptide. Polypeptides which are only capable of catalyzing the above-described reaction under certain, non-natural conditions, or which are only capable thereof to a very limited extent in comparison with the reaction which they catalyze predominantly, are not comprised.

The term "active fragment" as used in the present context describes nucleic acids which code for mevalonate kinase and which, while no longer complete, still code for polypeptides with the enzymatic activity of a mevalonate kinase and which are capable of catalyzing a reaction as described above which is characteristic of mevalonate kinase. Such fragments are shorter than the above-described complete nucleic acids which code for mevalonate kinase. In this context, nucleic acids may have been removed both at the 3′ and/or 5′ ends of the sequence, or else parts of the sequence which have no substantial adverse effect on the biological activity of mevalonate kinase may have been deleted, i.e. removed. A lower or else, if appropriate, increased activity which still permits the characterization or use of the resulting mevalonate kinase fragment is understood in this context as being sufficient within the context of the term used herein. The term "active fragment" can likewise refer to the amino acid sequence of the mevalonate kinase, in which case, analogously to what has been said above, it applies to those polypeptides which no longer comprise certain parts in comparison with the above-defined complete sequence, but where the enzymatic activity of the enzyme is not substantially adversely affected. The fragments can have different lengths.

The term "mevalonate kinase inhibition assay" or "inhibition assay" as used in the present context refers to a method or an assay which permits the recognition of the inhibition of the enzymatic activity of a polypeptide with the enzymatic activity of a mevalonate kinase as the result of one or more chemical compounds (candidate compound(s)), whereby the chemical compound can be identified as a mevalonate kinase inhibitor, or a fungicide.

The term "gene" as used in the present context is the name for a segment from the genome of a cell, which segment is responsible for the synthesis of a polypeptide chain.

The term "fungicide" or "fungicidal" as used in the present context refers to chemical compounds which are suitable for controlling fungi, in particular phytopathogenic fungi. Such phytopathogenic fungi are detailed hereinbelow, the enumeration not being limiting:

Plasmodiophoromycetes, Oomycetes, Chytridiomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes, for example,

Pythium species such as, for example, *Pythium ultimum*, Phytophthora species such as, for example, *Phytophthora infestans*, Pseudoperonospora species such as, for example, *Pseudoperonospora humuli* or *Pseudoperonospora cubensis*, Plasmopara species such as, for example, *Plasmopara viticola*, Bremia species such as, for example, *Bremia lactucae*, Peronospora species such as, for example, *Peronospora pisi* or *P. brassicae*, Erysiphe species such as, for example, *Erysiphe graminis*, Sphaerotheca species such as, for example, *Sphaerotheca fuliginea*, Podosphaera species such as, for example, *Podosphaera leucotricha*, Venturia species such as, for example, *Venturia inaequalis*, Pyrenophora species such as, for example, *Pyrenophora teres* or *P. graminea* (conidial form: Drechslera, syn: Helminthosporium), Cochliobolus species such as, for example, *Cochliobolus sativus* (conidial form: Drechslera, syn: Helminthosporium), Uromyces species such as, for example, *Uromyces appendiculatus*, Puccinia species such as, for example, *Puccinia recondita*, Sclerotinia species such as, for example, *Sclerotinia sclerotiorum*, Tilletia species such as, for example, *Tilletia caries*; Ustilago species such as, for example, *Ustilago nuda* or *Ustilago avenae*, Pellicularia species such as, for example, *Pellicularia sasakii*, Pyricularia species such as, for example, *Pyricularia oryzae*, Fusarium species such as, for example, *Fusarium culmorum*, Botrytis species, Septoria species such as, for example, *Septoria nodorum*, Leptosphaeria species such as, for example, *Leptosphaeria nodorum*, Cercospora species such as, for example, *Cercospora canescens*, Alternaria species such as, for example, *Alternaria brassicae* or Pseudocercosporella species such as, for example, *Pseudocercosporella herpotrichoides*.

Also of particular interest are, for example, *Magnaporthe grisea, Cochliobulus heterostrophus, Nectria hematococcus* and Phytophtora species.

Fungicidal active ingredients which are found with the aid of the mevalonate kinase according to the invention can, however, also interact with mevalonate kinases from fungal species which are pathogenic to humans, where the interaction with the different mevalonate kinases found in these fungi need not always be equally pronounced.

The subject matter of the present inventions is therefore also a method of identifying antimycotics, i.e. of mevalonate kinase inhibitors from fungi which are pathogenic to humans or animals and

which can be used for the preparation of compositions for treating diseases caused by fungi which are pathogenic to humans or animals.

Fungi which are pathogenic to humans and which are of particular interest in this context are the following, which can cause the symptoms described hereinbelow, inter alia:

Dermatophytes such as, for example, Trichophyton spec., Microsporum spec., *Epidermophyton floccosum* or *Keratomyces ajelloi*, which cause, for example, foot mycoses (tinea pedis),

Yeasts such as, for example, *Candida albicans*, which causes candidal esophagitis and dermatitis, *Candida glabrata, Candida krusei* or *Cryptococcus neoformans,* which may cause, for example, pulmonal cryptococcosis or else torulosis,

Molds such as, for example, *Aspergillus fumigatus*, *A. flavus, A. niger*, which cause, for example, bronchopulmonary Aspergillosis or fungal sepsis, Mucor spec., Absidia spec., or Rhizopus spec., which cause, for example, zygomycoses (intravasal mycoses), *Rhinosporidium seeberi*, which causes, for example, chronic granulomatous pharyngitis and tracheitis, *Madurella myzetomatis*, which causes, for example, subcutaneous mycetomas, *Histoplasma capsulatum*, which causes, for example, reticuloendothelial cytomycosis and Darling's disease, *Coccidioides immitis*, which causes, for example, pulmonary coccidioidomycosis and sepsis, *Paracoccidioides brasiliensis*, which causes, for example, South American blastomycosis, *Blastomyces dermatitidis*, which causes, for example, Gilchrist's disease and North American blastomycosis, *Loboa loboi,* which causes, for example, keloid blastomycosis and Lobo's disease, and *Sporothrix schenckii*, which causes, for example, sporotrichosis (granulomatous dermal mycosis).

The terms "fungicidal" and "fungicide" are hereinbelow understood as synonymously meaning the term "antimycotic" (adjective and noun), as well as the terms, respectively, "fungicidal" and "fungicide" in the traditional sense, i.e. referring to phytopathogenic fungi.

Fungicidal active ingredients which are found with the aid of a mevalonate kinase obtained from a particular fungus, in the present case for example from *U. maydis*, can therefore also interact with a mevalonate kinase from a large number of other fungal species, precisely also with other phytopathogenic fungi, where the interaction with the different mevalonate kinases which are found in these fungi need not always be equally pronounced. This explains, inter alia, the observed selectivity of the substances which affect this enzyme.

The term "competitor" as used in the present context refers to the characteristic of the compounds of competing with other compounds which are optionally to be identified for the binding to the mevalonate kinase and of displacing these compounds from the enzyme or being displaced thereby.

The term "agonist" as used in the present context refers to a molecule which accelerates or enhances the activity of mevalonate kinase.

The term "antagonist", or else "inhibitor" as used in the present context refers to a molecule which slows down or hinders the activity of mevalonate kinase.

The term "modulator" as used in the present context is the generic term for agonist and antagonist. Modulators can be small organochemical molecules, peptides or antibodies which bind to the polypeptides according to the invention or influence their activity. Furthermore, modulators can be small organochemical molecules, peptides or antibodies which bind to a molecule which, in turn, binds to the polypeptides according to the invention and thus influences their biological activity. Modulators can be natural substrates and ligands or else structural or functional mimetics of these. The term "modulator" as used in the present context is, however, preferably a molecule which is not the natural substrate or ligand.

Description of the invention

Mevalonate kinase (EC 2.7.1.36), hereinbelow also abbreviated to MK, catalyzes the reaction of mevalonate and adenosine triphosphate to give phosphomevalonate and adenosine diphosphate (Figure 1).

The reaction catalyzed by mevalonate kinase is an essential step at the beginning of ergosterol, dolichol or ubiquinone biosynthesis (Lees et al., 1997, Biochemistry and molecular biology of sterol synthesis in *Saccharomyces cerevisiae*. *Biochemistry and Function of Sterols*, 85-99; Mercer, 1984, The biosynthesis of ergosterol. *Pestic. Sci.* 15(2), 133-55; Karst and Lacroute, 1977, Ergosterol Biosynthesis in Saccharomyces cerevisiae. *Mol.Gen.Genet.* 154, 269-277).

Genes coding for mevalonate kinase have already been identified in a variety of fungi: in the yeasts *Saccharomyces cerevisiae* (Swissprot Accession No.: X55875) and *Schizosaccharomyces pombe* (Swissprot Accession No.: AB000541), or in *Neurospora crassa* (Swissprot Accession No.: NCB11N2). Sequence fragments of the gene coding for mevalonate kinase are known from the phytopathogenic fungi *Magnaporthe grisea* and *Fusarium sporotrichioides*. Besides, mevalonate kinase has also been found in a large number of other organisms, thus, for example, in *Homo sapiens* (Swissprot: Accession No.: AK023087), *Mus musculus* (Swissprot: Accession No.: BC005606) or *Oryza sativa* (Swissprot: Accession No.: AC091749).

The sequence similarities between different MKs within the classes of the eukaryotes are significant, while the sequence identity with the bacterial enzymes is less significant.

Mevalonate kinase was also isolated, from different organisms expressed, purified and characterized. (Tanaka et al., 1990, Purification and regulation of mevalonate kinase from rat liver *J. Biol. Chem.* 265(4), 2391-98; Chu, Xiusheng and Li, Ding, 2003, Cloning, expression, and purification of His-tagged rat mevalonate kinase. *Prot. Exp. Purific.* 27, 165-70; Schulte et al., 2000, Purification and characterization of mevalonate kinase from suspension-cultured cells of Catharanthus roseus (L.) G. Don. *Arc. Biochem. Biophys.* 378(2), 287-298; Oulmouden and Karst, 1991, Nucleotide sequence of the ERG12 gene of *Saccharomyces cerevisiae* encoding mevalonate kinase. Curr. Genet. 19, 9-14).

The present invention now provides for the first time the complete sequence of a mevalonate kinase from the phytopathogenic fungus *Ustilago maydis*, which makes possible the further study of mevalonate kinases, in particular from phytopathogenic fungi, and thus makes available a novel target protein for identifying novel fungicidal active compounds.

Despite extensive research into mevalonate kinase, it was hitherto unknown that mevalonate kinase can be a target protein for fungicidally active substances in fungi.

No fungicidal activity has been described for mevalonate kinase inhibitors which are already known (see, for example, Hinson et al., 1997, Post-translational regulation of mevalonate kinase by intermediates of the cholesterol and nonsterol isoprene biosynthetic pathways. *J. Lipid Res.* 38, 2216-2223; Tanaka et al., 1990). While various publication point out the particular role of mevalonate kinase (for example Oulmouden and Karst, 1991) for the metabolism of fungi, such as, for example, of the yeast fungus *Saccharomyces cerevisiae*, and also describe that the destruction of the yeast gene ERG12, which codes for mevalonate kinase, is lethal for *S. cerevisiae* (Oulmouden and Karst, 1990, Isolation of the *ERG12* gene of *Saccharomyces cerevisiae* encoding mevalonate kinase. Gene, 88, 253-257), none of the publications addresses the question of whether the enzyme mevalonate kinase can be influenced, for example inhibited, by active substances and whether a treatment of fungi *in vivo* with an active substance which modulates mevalonate kinase is possible in order to control these fungi. Mevalonate kinase has thus not been described to date as target protein for fungicides. No active substances which have a fungicidal activity and whose target is mevalonate kinase are known.

Thus, the present invention shows for the first time that mevalonate kinase not only constitutes an enzyme which is particularly important for fungi and thus especially suitable for being used as target protein for the search for further, improved fungicidally active active substances, but also that fungal mevalonate kinase can indeed be influenced by active substances *in vitro* and also *in vivo* and that these active substances can be employed as fungicides. Methods which can be used for identifying such fungicides are furthermore provided.

Within the scope of the present invention there has thus been developed a method which is suitable for determining the enzymatic activity of mevalonate kinase and the inhibition of this activity by one or more substances in what is known as an inhibition test and thus identifying modulators, preferably inhibitors of the enzyme, for example in HTS and UHTS methods. Inhibitors which have been identified and which display an inhibitory effect on a given mevalonate kinase even *in vitro* can then be tested for their fungicidal activity *in vivo*.

The inhibitors of a fungal mevalonate kinase can be used as fungicides in particular in crop protection or else as antimycotics in pharmacological indications. For example, the present invention shows that the inhibition of mevalonate kinase with a substance identified in a method according to the invention results in the dying or the damage of the treated fungi in synthetic media or on the plant.

Mevalonate kinase can be obtained from various fungi which are pathogenic or else humans or animals, for example from fungi such as the phytopathogenic fungus *U. maydis*. To obtain mevalonate kinase from fungi, the gene can, for example, be expressed recombinantly in *Escherichia coli* and an enzyme preparation can be prepared from *E. coli* cells (Example 1). It is preferred to employ mevalonate kinases from phytopathogenic fungi in order to identify fungicides which can be employed in crop protection. If the aim is to identify fungicides or antimycotics which are to be used in pharmacological indications, it is recommended to employ mevalonate kinase from fungi which are pathogenic to humans or animals.

To provide a mevalonate kinase from a phytopathogenic fungus, the relevant ORF (open reading frame) was amplified from genomic DNA via gene-specific primers by methods known to the skilled worker in order to express the mevalonate kinase UmErg12, which is encoded by *Umerg12* (*Um* stands for *Ustilago maydis*) as shown in SEQ ID NO:1. The corresponding DNA was cloned into the vector pDEST15 (Invitrogen, allows introduction of an N-terminal GST tag) in accordance with the manufacturer's instructions. The resulting plasmid pDEST15_umerg12 comprises the complete coding sequence of *Umerg12* in an N-terminal fusion with a GST tag from the vector. The UmErg12 fusion protein has a calculated mass of 74.5 kDa (cf. Example 1 and Figure 3).

The plasmid pDEST15_umerg12 was then used for recombinantly expressing UmErg12 in *E. coli* BL21 (DE3) (cf. Example 1).

The present invention therefore also provides a further, complete genomic sequence of a phytopathogenic fungus coding for a mevalonate kinase, and describes its use, or the use of the polypeptide encoded thereby, for identifying inhibitors of the enzyme and their use as fungicides.

The present invention therefore also relates to the nucleic acid, from the fungus *Ustilago maydis*, which codes for a polypeptide with the enzymatic function of a mevalonate kinase.

Mevalonate kinases are divided into homologous regions. Typical of mevalonate kinases is a conserved region which is involved in binding ATP.

This ATP binding site is a sequence feature which is characteristic of mevalonate kinases. Such a motif can be identified by a suitable search in the PROSITE database (Hofmann K., Bucher P., Falquet L., Bairoch A. (1999) "The PROSITE database, its status in 1999". *Nucleic Acids Res.* 27, 215). This can be shown as follows:

[LIVM]-[PK]-x-[GSTA]-x(0,1)-G-[LM]-[GS]-S-S-[GSA]-[GSTAC],

PROSITE allows the assignation of a function to polypeptides and thus makes it possible to recognize mevalonate kinases as such.

The Prosite motif is shown using the "one-letter code". The symbol "x" represents a position at which any amino acid is accepted. A variable position at which various specific amino acids are accepted is shown in "[...]", the amino acids which are possible at this position being enumerated. Amino acids which are not accepted at a specific position, in contrast, are shown in "{...}". A "-" separates the individual elements or positions of the motif. If a specific position is repeated, for example "x" several times in succession, this can be shown by showing the number of repetitions within brackets after the x, for example "x (3)", which represents "x-x-x".

Thus, a Prosite motif ultimately represents the components of a consensus sequence and distances between the amino acids involved, and is therefore typical of a particular class of enzymes. With reference to this motif, and based on the nucleic acids according to the invention, further polypeptides from phytopathogenic fungi which belong to the same class as the polypeptide according to the invention can be identified or assigned and which can thus also be used in accordance with the invention.

In the case of the *U. maydis* mevalonate kinase, this motif is likewise present in *S. cerevisiae, S. pombe, Magnaporthe grisea* or *N. crassa* (cf. Figure 2). The specific consensus sequence for a mevalonate kinase according to the invention which can be used for identifying or assigning further polypeptides according to the invention is therefore preferably

P-x-G-x-G-L-G-S-S-A,

and especially preferably the consensus sequence

P-I-G-A-G-L-G-S-S-A.

The abovementioned Prosite motif, or the specific consensus sequence, are typical of the polypeptides according to the invention whose structure can be defined with the aid of these consensus sequences and which are thereby also unambiguously identifiable.

The present invention therefore also relates to polypeptides from phytopathogenic fungi with the enzymatic activity of a mevalonate kinase, which comprise the abovementioned Prosite motif [LIVM]-[PK]-x-[GSTA]-x(0,1)-G-[LM]-[GS]-S-S-[GSA]-[GSTAC], preferably those polypeptides which comprise the abovementioned motif P-x-G-x-G-L-G-S-S-A, and especially preferably those polypeptides which comprise the consensus sequence P-I-G-A-G-L-G-S-S-A.

Owing to the homologies which exist in species-specific nucleic acids coding for mevalonate kinases, it is also possible to identify, and use, mevalonate kinases from other phytopathogenic fungi to solve the above problem, i.e. they can likewise be used for identifying inhibitors of mevalonate kinase, which, in turn, can be used as fungicides in crop protection. However, it is also feasible to use another fungus which is not phytopathogenic, or its mevalonate kinase, or the sequence coding therefor, in order to identify fungicidally active mevalonate kinase inhibitors. Owing to the sequence herein as shown in SEQ ID NO: 1 and any primers derived therefrom and, if appropriate, with the aid of the above-shown Prosite motif, the skilled worker can obtain, from other (phytopathogenic) fungi, and identify, further nucleic acids which code for mevalonate kinases, for example using PCR. Such nucleic acids and their use in methods of identifying fungicidal active substances are considered as being comprised by the present invention.

The present invention especially preferably relates to the nucleic acids of SEQ ID NO: 1 which code for the *Ustilago maydis* mevalonate kinase and the nucleic acids which code for the polypeptide as shown in SEQ ID NO: 2 or active fragments thereof.

The nucleic acids according to the invention are, in particular, single-stranded or double-stranded deoxyribonucleic acids (DNA) or ribonucleic acids (RNA). Preferred embodiments are fragments of genomic DNA, and cDNAs.

The nucleic acids according to the invention especially preferably comprise a sequence selected from among:

a) a sequence as shown in SEQ ID NO: 1,

b) sequences which code for a polypeptide which comprises the amino acid sequence as shown in SEQ ID NO: 2, and

c) sequences which have at least 90% identity with the sequences as defined under a) and b) and which code for the sequence motif [LIVM]-[PK]-x-[GSTA]-x(0,1)-G-[LM]-[GS]-S-S-[GSA]-[GSTAC].

As already specified above, the present invention is not only limited to the use of mevalonate kinase from *U. maydis*. Polypeptides with the enzymatic activity of a mevalonate kinase which polypeptides can then be employed in a method according to the invention can also be used or obtained analogously and in a manner known to the skilled worker from other fungi, preferably from phytopathogenic fungi. It is preferred to use the mevalonate kinase from *U. maydis*.

The present invention furthermore relates to DNA constructs which comprise a nucleic acid according to the invention and a homologous or heterologous promoter.

The term "homologous promoter" as used in the present context refers to a promoter which controls the expression of the gene in question in the starting organism. The term "heterologous promoter" as used in the present invention refers to a promoter whose characteristics differ from the promoter which controls the expression of the gene in question in the starting organism.

The choice of heterologous promoters depends on whether prokaryotic or eukaryotic cells or cell-free systems are used for the expression. Examples of heterologous promoters are the cauliflower mosaic virus 35S promoter for plant cells, the alcohol dehydrogenase promoter for yeast cells, and the T3, T7 or SP6 promoters for prokaryotic cells or cell-free systems.

Preferably, fungal expression systems such as, for example, the *Pichia pastoris* system should be used, transcription being driven in this case by the methanol-inducible AOX promoter.

The present invention furthermore relates to vectors which comprise a nucleic acid according to the invention, a regulatory region according to the invention or a DNA construct according to the invention. All of the phages, plasmids, phagemids, phasmids, cosmids, YACs, BACs, artificial chromosomes or particles which are suitable for particle bombardment which are used in molecular biological laboratories can be used as vectors.

Examples of preferred vectors are the p4XXprom. vector series (Mumberg, D., Müller, R., Funk, M.,.1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119-122) for yeast cells, pSPORT vectors (Life Technologies) for bacterial cells, or the Gateway vectors (Life Technologies) for various expression systems in bacterial cells, plants, *P. pastoris, S. cerevisiae* or insect cells.

The present invention also relates to host cells which comprise a nucleic acid according to the invention, a DNA construct according to the invention or a vector according to the invention.

The term "host cell" as used in the present context refers to cells which do not naturally contain the nucleic acids according to the invention.

Host cells which are suitable are prokaryotic cells, preferably *E. coli*, and eukaryotic cells, such as cells of *Saccharomyces cerevisiae, Pichia pastoris*, insects, plants, frog oocytes and mammalian cell lines.

The present invention furthermore relates to polypeptides with the enzymatic activity of a mevalonate kinase which are encoded by the nucleic acids according to the invention.

Preferably, the polypeptides according to the invention comprise an amino acid sequence selected from

(a) the sequence as shown in SEQ ID NO: 2,

(b) sequences which have a 95% identity with the sequence defined under a), and sequence motif [LIVM]-[PK]-x-[GSTA]-x(0,1)-G-[LM]-[GS]-S-S-[GSA]-[GSTAC], and

(c) fragments of the sequences stated under a) and b) which have the same enzymatic activity as the sequence defined under a).

The term "polypeptides" as used in the present context refers not only to short amino acid chains which are generally referred to as peptides, oligopeptides or oligomers, but also to longer amino acid chains which are normally referred to as proteins. It comprises amino acid chains which can be modified either by natural processes, such as post-translational processing, or by chemical prior-art methods. Such modifications may occur at various sites and repeatedly in a polypeptide, such as, for example, on the peptide backbone, on the amino acid side chain, on the amino and/or the carboxyl terminus. For example, they comprise acetylations, acylations, ADP ribosylations, amidations, covalent linkages to flavins, heme moieties, nucleotides or nucleotide derivatives, lipids or lipid derivatives or phophatidylinositol, cyclizations, disulfide bridge formations, demethylations, cystine formations, formylations, gamma-carboxylations, glycosylations, hydroxylations, iodinations, methylations, myristoylations, oxidations, proteolytic processings, phosphorylations, selenoylations and tRNA-mediated amino acid additions.

The polypeptides according to the invention may exist in the form of "mature" proteins or as parts of larger proteins, for example as fusion proteins. They can furthermore exhibit secretion or leader sequences, pro-sequences, sequences which allow simple purification, such as polyhistidine residues, or additional stabilizing amino acids. The proteins according to the invention may also exist in the form in which they are naturally present in their source organism, from which they can be obtained directly, for example. Likewise, active fragments of a mevalonate kinase may be employed in the methods according to the invention, as long as they make possible the determination of the enzymatic activity of the polypeptide, or its inhibition by a candidate compound.

In comparison with the corresponding regions of naturally occurring mevalonate kinases, the polypeptides used in the methods according to the invention can have deletions or amino acid substitutions, as long as they still exhibit at least the enzymatic activity of a complete mevalonate

kinase. Conservative substitutions are preferred. Such conservative substitutions comprise variations, one amino acid being replaced by another amino acid from the following group:

1. Small, aliphatic residues, which are non-polar or of little polarity: Ala, Ser, Thr, Pro and Gly;

2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;

3. Polar, positively charged residues: His, Arg and Lys;

4. Large, aliphatic, non-polar residues: Met, Leu, Ile, Val and Cys; and

5. Aromatic residues: Phe, Tyr and Trp.

One possible mevalonate kinase purification method is based on preparative electrophoresis, FPLC, HPLC (for example using gel filtration columns, reversed-phase columns or mildly hydrophobic columns), gel filtration, differential precipitation, ion-exchange chromatography or affinity chromatography (cf. Example 1).

A rapid method of isolating the mevalonate kinases which are synthesized by host cells starts with expressing a fusion protein, where the fusion moiety may be purified in a simple manner by affinity purification. For example, the fusion moiety may be a GST tag (cf. Example 1), in which case the fusion protein can be purified on glutathione-Sepharose column. The fusion moiety can be removed by partial proteolytic cleavage, for example at linkers between the fusion moiety and the polypeptide according to the invention which is to be purified. The linker can be designed in such a way that it includes target amino acids, such as arginine and lysine residues, which define sites for trypsin cleavage. Standard cloning methods using oligonucleotides may be employed for generating such linkers.

Other purification methods which are possible are based, in turn, on preparative electrophoresis, FPLC, HPLC (e.g. using gel filtration columns, reversed-phase columns or mildly hydrophobic columns), gel filtration, differential precipitation, ion-exchange chromatography and affinity chromatography.

The terms "isolation or purification" as used in the present context mean that the polypeptides according to the invention are separated from other proteins or other macromolecules of the cell or of the tissue. The protein content of a composition containing the polypeptides according to the invention is preferably at least 10 times, more preferably at least 100 times, higher than in a host cell preparation.

The polypeptides according to the invention may also be affinity-purified without fusion moieties with the aid of antibodies which bind to the polypeptides.

The method of preparing polypeptides with the enzymatic activity of a mevalonate kinase, such as, for example, the polypeptide UmErg12, is thus characterized in that

(a) a host cell comprising at least one expressible nucleic acid sequence coding for a fungal polypeptide with the enzymatic activity of a mevalonate kinase is cultured under conditions which ensure the expression of this nucleic acid, or

(b) an expressible nucleic acid sequence encoding a fungal polypeptide with the enzymatic activity of a mevalonate kinase is expressed in an *in-vitro* system, and

(c) the polypeptide is recovered from the cell, the culture medium or the *in-vitro* system.

The cells thus obtained which comprise the polypeptide according to the invention, or the purified polypeptide thus obtained, are suitable for use in methods of identifying mevalonate kinase modulators or inhibitors.

The present invention also relates to the use of polypeptides from fungi, preferably from fungi which are phytopathogenic or pathogenic to humans or animals, which have the enzymatic activity of a mevalonate kinase in methods of identifying inhibitors of these polypeptides, and to the use of these mevalonate kinase inhibitors as fungicides.

Fungicidal active compounds which are found with the aid of a mevalonate kinase from a specific fungal species can also interact with mevalonate kinases from other fungal species, but the interaction with the different mevalonate kinases which are present in these fungi need not always be equally pronounced. This explains inter alia the selectivity of active substances. The use in other fungal species of active compounds which have been found with a mevalonate kinase of specific fungal species, can be attributed to the fact that mevalonate kinases from different fungal species are very closely related and show pronounced homology over substantial regions. Thus, it is clear from Figure 2 that such a homology over substantial sequence segments exists between *U. maydis*, *S. cerevisiae, N. crassa, S. pombe* and *M. grisea* and that, therefore, the effect of the substances found with the aid of *U. maydis* mevalonate kinase is not limited to *U. maydis*. This is why, in methods of identifying fungicides, it is preferred to use polypeptides with the enzymatic activity of a mevalonate kinase which have a consensus sequence as shown in Figure 2.

Methods which are suitable for identifying modulators, in particular inhibitors or antagonists, of the polypeptides according to the invention are generally based on the determination of the activity or the biological functionality of the polypeptide. Suitable for this purpose are, in principle, methods based on intact cells (*in-vivo* methods), but also methods which are based on the use of the polypeptide isolated from the cells, which may be present in purified or partially purified form or else as a crude extract. These cell-free *in-vitro* methods, like *in-vivo* methods, can be used on a laboratory scale, but preferably also in HTS or UHTS methods. Following the *in-vivo* or *in-vitro*-identification of modulators of the polypeptide, fungal cultures can be assayed in order to test the fungicidal activity of the compounds which have been found.

A large number of assay systems for the purpose of assaying compounds and natural extracts are preferably designed for high throughput numbers in order to maximize the number of substances assayed within a given period. Assay systems based on cell-free processes require purified or semipurified protein. They are suitable for an "initial" assay, which aims mainly at detecting a possible effect of a substance on the target protein. Once such an initial assay has taken place, and one or more compounds, extracts and the like have been found, the effect of such compounds can be studied in the laboratory in a more detailed fashion. Thus, inhibition or activation of the polypeptide according to the invention *in vitro* can be assayed again as a first step in order to subsequently assay the activity of the compound on the target organism, in this case one or more phytopathogenic fungi. If appropriate, the compound can then be used as starting point for the further search and development of fungicidal compounds which are based on the original structure, but are optimized with regard to, for example, activity, toxicity or selectivity.

To find modulators, for example a synthetic reaction mix (for example *in-vitro* transcription products) or a cellular component such as a membrane, a compartment or any other preparation comprising the polypeptides according to the invention can be incubated together with an optionally labelled substrate or ligand of the polypeptides in the presence and absence of a candidate molecule, which may take the form of an antagonist. The ability of the candidate molecule to inhibit the activity of the polypeptides according to the invention can be identified for example on the basis of reduced binding of the optionally labelled ligand or a reduced conversion of the optionally labelled substrate. Molecules which inhibit the biological activity of the polypeptides according to the invention are good antagonists.

An example of a method by which modulators of the polypeptides according to the invention can be found is a displacement assay in which the polypeptides according to the invention and a potential modulator are combined, under suitable conditions, with a molecule which is known to bind to the polypeptides according to the invention, such as a natural substrate or ligand or a

substrate or ligand mimetic. The polypeptides according to the invention can themselves be labelled, for example fluorimetrically or colorimetrically, so that the number of the polypeptides which are bound to a ligand or which have undergone a conversion can be determined accurately. However, binding can likewise be monitored by means of the optionally labelled substrate, ligand or substrate analogue. The efficacy of the antagonist can be determined in this manner.

Effects such as cell toxicity are, as a rule, ignored in these *in-vitro* systems. The assay systems check not only inhibitory, or suppressive, effects of the substances, but also stimulatory effects. The efficacy of a substance can be checked by concentration-dependent assay series. Control mixtures without test substances or without enzyme can be used for assessing the effects.

Owing to the host cells which comprise nucleic acids coding for a mevalonate kinase according to the invention and which are available on the basis of the present invention, the development of cell-based assay systems for identifying substances which modulate the activity of the polypeptides according to the invention, is made possible.

The modulators to be identified are preferably small organochemical compounds.

A method of identifying a compound which modulates the activity of a fungal mevalonate kinase and which can be used in crop protection as fungicide preferably consists in

a) a polypeptide with the enzymatic activity of a mevalonate kinase, preferably from fungi and especially preferably from phytopathogenic fungi,

or a host cell comprising such a polypeptide, being brought into contact with a chemical compound or with a mixture of chemical compounds under conditions which permit the interaction of the chemical compounds with the polypeptide,

b) comparing the activity of the polypeptide in the absence of the chemical compound with the activity of the polypeptide in the presence of the chemical compound or mixture of the chemical compounds,

c) selecting the chemical compound which specifically modulates the activity of the polypeptide according to the invention, and, if appropriate,

d) subjecting the fungicidal activity of the compound selected to *in-vivo* tests.

In this context, it is particularly preferable to determine the compound which specifically inhibits the activity of the polypeptide according to the invention. The term "activity" as used in the present context refers to the biological activity of the polypeptide according to the invention.

A preferred embodiment exploits the fact that one adenosine diphosphate (ADP) molecule is liberated in the mevalonate kinase reaction. The activity, or the decrease or increase in activity, of the polypeptide according to the invention can thus be determined by detecting the ADP which is being formed. Here, the lower, or inhibited, activity of the polypeptide according to the invention is monitored with the aid of the detection of the ADP being formed, by coupling with the downstream reaction of pyruvate kinase and lactate dehydrogenase. By using ADP, pyruvate kinase converts phosphoenolpyruvate into pyruvate, which is then utilized by lactate dehydrogenase for oxidizing NADH to give NAD. The NAD concentration, which increases owing to the coupled reaction, or the NADH concentration, which decreases owing to the coupled reaction, can be measured photospectrometrically by absorption or fluorescence measurement (at 340 nm or an excitation wavelength of 360 nm and an emission wavelength of 465 nm).

The measurement can also be carried out in formats conventionally used for HTS or UHTS assays, for example in microtiter plates, into which for example a total volume of 5 to 50 µl is introduced per mixture or per well and the individual components are present in the desired final concentrations (cf. Example 2). The compound (candidate molecule) to be assayed and which potentially inhibits or activates the activity of the enzyme is introduced for example in a suitable concentration in assay buffer, comprising mevalonate, adenosine triphosphate, phosphoenolpyruvate and the coupled auxiliary enzymes pyruvate kinase and lactate dehydrogenase. The polypeptide according to the invention is then added in assay buffer, thus starting the reaction. The mixture is then incubated at a suitable temperature, and the decrease in absorption is measured at 340 nm. The incubation time here can be varied over a substantial period. Preferably, incubation is carried out for 5 to 60, preferably for 15 to 45 minutes, that is to say on average approximately 30 minutes. The coupled enzyme assay is described, for example, in Tanaka et al., 1990.

A further measurement is carried out in a corresponding mixture, but without addition of a candidate molecule and without addition of a polypeptide according to the invention (negative control). Another measurement, in turn, is carried out in the absence of a candidate molecule, but in the presence of the polypeptide according to the invention (positive control). The negative and the positive controls thus provide the comparative values for the mixtures in the presence of a candidate molecule.

To determine optimal conditions for a method of identifying mevalonate kinase inhibitors or for determining the activity of the polypeptides according to the invention, it may be advantageous to determine the respective K_M value of the polypeptide according to the invention used. This value then provides information on the concentration of the substrate(s) to be used by preference. In the

case of *U. maydis* mevalonate kinase, a K_M of 130 µM was determined for adenosine triphosphate and a K_M of 200 µM for mevalonate.

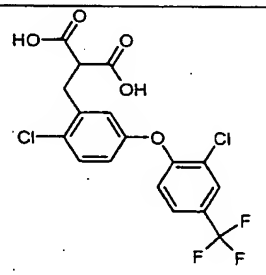
Compounds which inhibit fungal mevalonate kinase and which are capable of damaging or destroying different fungal species (for example growth inhibition) were successfully identified within the scope of the present invention with the aid of the methods which have been described above by way of example.

In addition to the abovementioned methods of determining the enzymatic activity of a mevalonate kinase or the inhibition of this activity and of identifying fungicides, other methods or inhibition tests, for example methods or inhibition tests which are already known, can, of course, also be used as long as these methods allow the determination of the activity of a mevalonate kinase and the detection of an inhibition of this activity by a candidtate compound. An alternative method is based for example on the use of radiolabelled ATPs (Oulmouden und Karst, 1991).

In this manner, mevalonate kinase inhibitors have been identified using the method according to the invention.

Table I shows an example of a compound which has been successfully identified as a mevalonate kinase inhibitor using a method according to the invention.

Table I

Example	Compound
1	

It has furthermore been shown within the scope of the present invention that the inhibitors of a mevalonate kinase according to the invention which have been identified with the aid of a method according to the invention are capable of damaging or destroying fungi.

To this end, a solution of the active compound to be tested may be pipetted for example into the wells of microtiter plates. After the solvent has evaporated, medium is added to each well. The medium is previously treated with a suitable concentration of spores or mycelium of the test

fungus. The resulting concentrations of the active compound are, for example, 0.1, 1, 10 and 100 ppm.

The plates are subsequently incubated on a shaker at a temperature of about 22°C until sufficient growth is discernible in the untreated control.

The plates are evaluated photometrically at a wavelength of 620 nm. The dose of active compound which leads to a 50% inhibition of the fungal growth over the untreated control (ED_{50}) can be calculated from the readings of the different concentrations. In this manner it was demonstrated that mevalonate kinase inhibitors which have a fungicidal *in vivo* activity have successfully been identified using a method according to the invention.

Table II shows examples of the results of such a test for a compound found in a method according to the invention in the form of ED_{50} values (cf. Table I). The compound has a fungicidal activity against different fungi.

Table II

Compound (Ex.)	Organism	ED_{50} [ppm]
1	*Botrytis cinerea*	0.37
1	*Pyricularia oryzae*	<0.10
1	*Aspergillus nidulans*	0.28

The present invention therefore also relates to the use of modulators of fungal mevalonate kinase, preferably of mevalonate kinase from phytopathogenic fungi, as fungicides and to methods of controlling fungal attacks in plants by allowing an inhibitor of a fungal mevalonate kinase to act on the fungus or on the plant which is attacked by the fungus.

The present invention also relates to fungicides which have been identified with the aid of a method according to the invention.

Compounds which are identified with the aid of a method according to the invention and which, owing to inhibition of the fungal mevalonate kinase, are fungicidally active can thus be used for the preparation of fungicidal compositions.

Depending on their respective physical and/or chemical characteristics, the active compounds which have been identified can be converted into the customary formulations, such as solutions, emulsions,

suspensions, powders, foams, pastes, granules, aerosols, very fine capsules in polymeric substances and in coating compositions for seed and also ULV cold- and hot-fogging formulations.

These formulations are produced in a known manner, for example by mixing the active compounds with extenders, that is, liquid solvents, liquefied gases under pressure, and/or solid carriers, optionally with the use of surfactants, that is, emulsifiers and/or dispersants and/or foam-formers. In the case of the use of water as an extender, organic solvents can, for example, also be used as cosolvents. As liquid solvents, there are suitable in the main: aromatics, such as xylene, toluene or alkylnaphthalenes, chlorinated aromatics or chlorinated aliphatic hydrocarbons, such as chlorobenzenes, chloroethylenes or methylene chloride, aliphatic hydrocarbons, such as cyclohexane or paraffins, for example mineral oil fractions, alcohols, such as butanol or glycol as well as their ethers and esters, ketones, such as acetone, methyl ethyl ketone, methyl isobutyl ketone or cyclohexanone, strongly polar solvents, such as dimethylformamide and dimethyl sulfoxide, and water. By liquefied gaseous extenders or carriers are meant liquids which are gaseous at ambient temperature and under atmospheric pressure, for example aerosol propellants, such as halogenohydrocarbons and butane, propane, nitrogen and carbon dioxide. As solid carriers there are suitable: for example ground natural minerals, such as kaolins, clays, talc, chalk, quartz, attapulgite, montmorillonite or diatomaceous earth, and ground synthetic minerals, such as highly disperse silica, alumina and silicates. As solid carriers for granules there are suitable: for example crushed and fractionated natural rocks such as calcite, marble, pumice, sepiolite and dolomite, as well as synthetic granules of inorganic and organic meals, and granules of organic material such as sawdust, coconut shells, maize cobs and tobacco stalks. As emulsifiers and/or foam-formers there are suitable: for example nonionic and anionic emulsifiers, such as polyoxyethylene fatty acid esters, polyoxyethylene fatty alcohol ethers, for example alkylaryl polyglycol ethers, alkylsulfonates, alkyl sulfates, arylsulfonates and protein hydrolysates. As dispersants there are suitable: for example lignin-sulfite waste liquors and methylcellulose.

Adhesives such as carboxymethylcellulose and natural and synthetic polymers in the form of powders, granules or latices, such as gum arabic, polyvinyl alcohol and polyvinyl acetate, as well as natural phospholipids, such as cephalins and lecithins, and synthetic phospholipids can be used in the formulations. Further additives may be mineral and vegetable oils.

It is possible to use colorants such as inorganic pigments, for example iron oxide, titanium oxide and Prussian Blue, and organic dyestuffs, such as alizarin dyestuffs, azo dyestuffs and metal phthalocyanine dyestuffs, and trace nutrients such as salts of iron, manganese, boron, copper, cobalt, molybdenum and zinc.

The formulations generally comprise between 0.1 and 95 per cent by weight of active compound, preferably between 0.5 and 90%.

The active compounds according to the invention, as such or in their formulations, can also be used as a mixture with known fungicides, bactericides, acaricides, nematicides or insecticides, for example in order to widen in this way the spectrum of action or to prevent the build-up of resistance. In many cases, synergistic effects are achieved, i.e. the efficacy of the mixture exceeds the efficacy of the individual components.

When employing the compounds according to the invention as fungicides, the application rates can be varied within substantial ranges, depending on the application.

All plants and plant parts may be treated in accordance with the invention. In the present context, plants are understood as meaning all plants and plant populations, such as desired and undesired wild plants or crop plants (including naturally occurring crop plants). Crop plants may be plants which can be obtained by traditional breeding and optimization methods or by biotechnological and recombinant methods or combinations of these methods, including the transgenic plants and including those plant varieties which are capable, or not capable, of protection by Plant Breeders' Rights. Plant parts are to be understood as meaning all aerial and subterranean parts and organs of the plants, such as shoot, leaf, flower and root, examples which are mentioned being leaves, needles, stems, stalks, flowers, fruiting bodies, fruits and seeds, and also roots, tubers and rhizomes. The plant parts also include harvested material and vegetative and generative propagation material, for example cuttings, tubers, rhizomes, slips and seeds.

The treatment according to the invention of the plants and plant parts with the active compounds is effected directly or by acting on their environment, habitat or store by the customary treatment methods, for example by dipping, spraying, vaporizing, fogging, scattering, brushing on and, in the case of propagation material, in particular seeds, furthermore by coating with one or more coats.

The examples which follow illustrate various aspects of the present invention and are not to be construed as limiting.

Examples

Example 1

Cloning, expression and purification of *umerg12* and UmErg12, respectively, from *Ustilago maydis*

To clone and express *Umerg12*, the ORF from genomic DNA from *Ustilago maydis* was amplified via gene-specific primers. The corresponding DNA, a 1059 bp amplicon, was first cloned into the vector pDON201 from Invitrogen and subsequently cloned into the vector pDEST15 (Invitrogen) via recombination. The resulting plasmid pDEST15_umerg12 comprises the complete coding sequence of *umerg12* in an N-terminal fusion with the GST tag, which is part of the vector. The UmErg12 fusion protein has a calculated mass of 74.5 kDa.

For the heterologous expression, the plasmid pDEST15_umerg12 was transformed into *E. coli* BL21 (DE3). A preculture of the transformants was inoculated into 50 ml selection medium. These cells were incubated overnight at 37°C and subsequently diluted 1:20 in selection medium (LB medium supplemented with 100 µg/ml ampicillin). Induction was effected at an OD_{600nm} of 0.8 using 0.1 mM IPTG (final concentration) at 30°C. After 3 hours of induction, the cells were harvested and processed directly.

Disruption was carried out by sonication in lysis buffer (100 mM Tris-HCl, pH 8, 5 mM DTT, 5 mM $MgCl_2$, 0.1% Triton, 10 µM ATP) after previously having been treated with lysozyme (15 minutes, 30°C, 1 mg/ml final concentration in lysis buffer). The cytoplasm fraction obtained by centrifugation (15 minutes at 4°C, 10 000 g) was used for the purification of the expressed protein. After the mixture had been filtered through a 0.45 µm Nalgene filtration unit, purification was carried out in accordance with the standard protocol of the manufacturer for glutathion-Sepharose columns using the following running buffer (100 mM Tris/HCl, pH 7.5; 10 µM ATP, 5 mM $MgCl_2$, 5 mM DTT, 10% glycerol). The elution buffer used was 50 mM Tris/HCl pH 8.0, 5 mM DTT, 5 mM $MgCl_2$, 10 µM ATP, 10% glycerol supplemented with 20 mM reduced glutathione. The purified protein was stored at –80°C. 250 ml of culture medium yielded approximately 2.5 mg of soluble protein which was ready for use in methods of identifying mevalonate kinase modulators.

Example 2

(A) Identification of mevalonate kinase modulators in 384-Well MTPs in a coupled assay

384-well microtiter plates from Greiner were used for the identification of mevalonate kinase modulators.

The negative control was pipetted into the first and second row. The negative control was composed of 5 µl of solution 1 (5% DMSO in H_2O), 20 µl of solution 2 (100 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 12.5 mM glutathione, 0.25% BSA) and 25 µl of solution 3 (100 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 20 mM KCl, 0.6 mM ATP, 0.8 mM PEP, 0.6 mM NADH, 20 mM DTT, 0.02% Tween 20) supplemented with 200 mU pyruvate kinase and 400 mU lactate dehydrogenase.

The positive control was pipetted into the third and fourth row. The positive control was composed of 5 µl of solution 1 (5% DMSO in H_2O), 20 µl of solution 4 (100 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 12.5 mM glutathione, 0.25% BSA, 0.1 µg mevalonate kinase) and 25 µl of solution 3 (100 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 20 mM KCl, 0.6 mM ATP, 0.8 mM PEP, 0.6 mM NADH, 20 mM DTT, 0.02% Tween 20) supplemented with 200 mU pyruvate kinase and 400 mU lactate dehydrogenase.

A test substance in a concentration of 2 µM in DMSO was introduced into the remaining rows, H_2O being used for diluting the substance. After addition of 20 µl of solution 4 (100 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 12.5 mM glutathione, 0.25% BSA, 0.1 µg mevalonate kinase), 25 µl of solution 3 (100 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 20 mM KCl, 0.6 mM ATP, 0.8 mM PEP, 0.6 mM NADH, 20 mM DTT, 0.02% Tween 20) supplemented with 200 mU pyruvate kinase and 400 mU lactate dehydrogenase were added to initiate the reaction. This was followed by incubation at 25°C for 20 minutes. The measurement which followed was carried out by determining the absorption at 340 nm in a Tecan SPECTRAFluor Plus which is suitable for MTP.

Example 3

Demonstration of the fungicidal activity of the mevalonate kinase inhibitors identified

A methanolic solution of the active substance identified with the aid of a method according to the invention in a suitable amount, treated with an emulsifier, was pipetted into the wells of microtiter plates. After the solvent had evaporated, 200 µl of potato dextrose medium were added to each well. The medium had previously been treated with suitable concentrations of spores or mycelia of the test fungus.

The resulting emulsifier concentration was 300 ppm.

The plates were subsequently incubated on a shaker at a temperature of 22°C until sufficient growth was observed in the untreated control. Evaluation was carried out photometrically at a wavelength of 620 nm. The dose of active substance which results in a 50% inhibition of the fungal growth over the untreated control (ED_{50}) is calculated from the readings of the different concentrations.

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